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PREVALENCE AND PRODUCTION OF ENZYMES BY *Candida* ISOLATES FROM VAGINAL SECRETION SAMPLES

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ABSTRACT

Vulvovaginal candidiasis (VVC) ranks second among causes of vaginitis. *Candida albicans* is responsible for the majority of symptomatic episodes of VVC. However, one fact to consider is the increase of infections by species of non-*C. albicans*, which contribute to high rates of recurrence and resistance. The purpose of this study was to isolate *Candida* spp. samples from vaginal discharge, determine the prevalence of the species and assess the production of hydrolytic enzymes (proteases, phospholipases, hemolysins, catalase, and gelatinases) in clinical isolates. 144 samples from patients treated at a public hospital in São Luis-MA were analyzed. The production of hydrolytic enzymes was determined in triplicate with specific methods. Statistical analysis was performed using the x^2, Kruskal-Wallis and Student-Newman-Keuh tests. Ninety patients had positive cultures for *Candida* spp. *Candida parapsilosis* was the main isolated species (43.3%). Forty-three patients (47.8%) showed clinical manifestations that suggested VVC. The correlation between the presence of *Candida* species and the presence or absence of symptoms was not statistically significant ($x = 3.22$, $p = 0.073$). The expression of enzymes by *Candida* spp. isolates was recorded with percentages: hemolysins (80%), phospholipases (8.9%), proteinases (17.8%), catalase (64.4%) and gelatinases (10.0%). Statistically significant differences were observed among the isolates for expression of phospholipases ($p=0.0005$), with *C. albicans* being the largest producer and for catalase ($p=0.0045$) with *C. parapsilosis* and *C. albicans* the species with increased expression. This study shows a predominance of non-*albicans* species in vulvovaginal samples and most of the isolates were producers of hemolysin and catalase, which may contribute to the virulence of the strains of *Candida* spp. and the development of vaginal infection under appropriate conditions.

KEY WORDS: *Candida* spp; vulvovaginitis; virulence factors.

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RESUMO

Prevalência e produção de enzimas por isolados de *Candida* provenientes de amostras de secreção vaginal

A candidíase vulvovaginal (VVC) ocupa o segundo lugar entre as vaginites, sendo *Candida albicans* responsável pela maioria dos episódios sintomáticos de VVC. Entretanto, um fato a ser considerado é o aumento de infecções por espécies de *C.* não *albicans*, o que contribui para as elevadas taxas de recidiva e resistência. Os objetivos deste estudo foram isolar *Candida* spp. de amostras de secreção vaginal, determinar a prevalência das espécies e verificar a produção de enzimas hidrolíticas (proteases, fosfolipases, hemolisinas, catalases e gelatinases) nos isolados clínicos. Foram analisadas 144 amostras de pacientes atendidas em um hospital público em São Luís-MA. A produção de enzimas hidrolíticas foi conduzida em triplicata usando-se métodos específicos. A análise estatística foi realizada por meio dos testes x^2, Kruskal-Wallis e Student-Newman-Keuh. Apresentaram cultura positiva para *Candida* spp. 90 pacientes. A principal espécie isolada (43,3%) foi *Candida parapsilosis* e 43 pacientes (47,8%) apresentaram manifestações clínicas sugestivas de VVC. A correlação entre a presença de espécies de *Candida* e a presença ou ausência de sintomas não foi estatisticamente significante (x = 3,22 , p = 0,073). Verificou-se a expressão de enzimas pelos isolados de *Candida* spp. nas seguintes porcentagens: hemolisinas (80%), fosfolipases (8,9%), proteinases (17,8%), catalases (64,4%) e gelatinases (10,0%). Diferenças estatisticamente significativas foram observadas entre os isolados em relação à expressão de fosfolipases (p = 0,0005), sendo *C. albicans* a espécie mais produtora, e de catalases (p = 0,0045), em que *C. parapsilosis* e *C. albicans* foram as espécies com expressão mais elevada. Este estudo mostrou uma predominância de espécies não *albicans* em amostras vulvovaginais e a maioria dos isolados revelou-se produtora de hemolisinas e catalases, o que pode contribuir para a virulência das linhagens de *Candida* e para o desenvolvimento de infecção vaginal quando em condições apropriadas.

DESCRITORES: *Candida* spp; vulvovaginite; fatores de virulência.

INTRODUCTION

During the development of preventative gynecological examinations, many women present with vulvovaginitis caused by *Candida* spp. (VVC), which is associated with unpleasant and uncomfortable signs and symptoms for most patients. VVC corresponds to a pathological process triggered by the abnormal growth of yeasts in the region of the female genital tract mucosa (2), which under favorable conditions may determine the emergence of an infectious process because of local or systemic factors (12). Possible signs and symptoms presented by this disease are itching, pain, swelling and redness (hyperemia) of the vulva and vagina, and a white secretion, resembling "buttermilk" (3). VVC cases, when accompanied by severe illness or illnesses that weaken the immune system, become aggravating and difficult to treat, causing relapses and likely causing systemic candidiasis (7, 16).

Despite the high prevalence of VVC (approximately 75% of women present at least one episode of VVC and 50% present 2 or more episodes during their lifetime), the diagnosis of *Candida* species is performed equivocally, which leads to incorrect therapy and the onset of relapses and resistance (22).

Candida albicans is responsible for the majority of symptomatic episodes of VVC and among the non-*albicans Candida* (NAC) species *Candida glabrata* is considered the most common (7, 40). A fact to consider is the increase in infections by NAC species (*Candida tropicalis, Candida glabrata, Candida parapsilosis, Candida krusei, Candida lusitaniae* and *Candida pseudotropicalis)* that contribute to high rates of recurrence and resistance, in such a way that *C. albicans,* in some populations*,* has been responsible for only approximately 50% of the cases (14). Thus, epidemiological data about risk factors and pathogenic mechanisms are still improperly studied. Although the treatment of uncomplicated cases is simple and associated with high cure rates, cases of recurrence and resistance continue to plague many women. With a better understanding of the pathophysiological processes for both conditions, better therapeutic approaches may be available for chronic patients in the near future.

A variety of factors, such as those inherent to the yeast and the host, are important in the development of VVC. In women where candidiasis is complicated, host and yeast factors distinguish the infection from an uncomplicated case. These factors have a profound impact on therapy. From the host's standpoint, prior colonization by yeast and a subsequent decrease in immune response, observed in immunosuppressive diseases, pregnant women and chronic users of corticosteroids, seem to favor the infection. Other contributing factors include the use of antibiotics, estrogen therapy, minor traumas such as sexual intercourse, habitual wearing of tight clothes or synthetic fibers and a very acidic diet (6).

The factors related to yeast are virulence factors, such as the ability to adhere, the production of hydrolytic enzymes and antifungal resistance presented by some species. The wide variety of candidiasis presentations and its clinical significance have stimulated the interest in studying the mechanisms of pathogenicity of *Candida* species and the identification of their virulence factors (32).

Among the yeast exoenzymes that may facilitate pathogenesis, the acidic aspartyl proteases and phospholipases are noteworthy (34). The acidic aspartate proteinases promote adhesion, invasion and tissue damage (24) whereas the phospholipases are related to the process of membrane rupture at the time of host cell invasion. Other hydrolytic enzymes also considered to be virulence factors are catalase and hemolysin. The intracellular catalase present in *Candida* yeasts has been associated with mechanisms of virulence, drug resistance and immunogenicity (23). There are some studies concerning proteases and phospholipases (44, 24, 34, 13), however little is known about hemolysin production in *Candida* species.

The ability of a microorganism to acquire iron from hemoglobin or hemin is relevant to its survival within the mammalian host (5). Hemolysins enable the pathogen to extract iron from those molecules or host cells (19).Hemolysin produced by pathogenic microorganisms is classified into two types: α and β (15).

The purpose of this study was to isolate *Candida* spp. samples from vaginal secretions, determine the prevalence of the species and assess the production

of hydrolytic enzymes (proteases, phospholipases, hemolysins, catalase, and gelatinases) in clinical isolates.

MATERIALS AND METHODS

Patients and Sample Collection

Swabs were collected from vaginal mucus of 144 patients that, regardless of the presence of symptoms of vulvovaginal candidiasis, spontaneously sought the gynecology service at the Women's Hospital in São Luís– MA, from October 2010 to May 2011. The swabs were stored in transport medium, under refrigeration (4°C) until the time of cultivation. The patients included in the study signed a consent form and were informed about the research purpose, as well as the guarantee of privacy and data reliability. This study was approved by the Ethics Committee on Human Research of the University of Ceuma, under protocol nr 267/10. Women who had used antifungal medication (oral and/or vaginal) in the last 30 days did not participate in the research.

Isolation and Identification of *Candida* spp.

The swabs of the vaginal mucosa were inoculated on to Petri plates containing Sabouraud Dextrose Agar with chloramphenicol and incubated at 37°C for 24 hours. The isolated colonies were identified through the medium CHROMagar Candida -AGAR HICROME CANDIDA (HiMed) and the automated method VITEK (bioMérieux, France). After isolation, all strains were maintained in Brain Heart Infusion (BHI) medium with 15% glycerol at -20°C (36).

Preparation of Inocula

The inoculum of yeast cells was made from stock cultures and incubated for 18 hours at 37ºC in BHI (Brain Heart Infusion – Acumedia Manufactures) or in liquid RPMI-1640 medium and standardized to approximately 106 UFC/ml according to the turbidity of the range 0.5 of McFarland (11).

Production of Hemolysin (hemolytic factor)

The production of the factor was assessed using the plaque method of analysis described by Luo, Samaranayake and Yau (19), considering some adjustments of Manns et al. (21). The yeast suspension (3µl) was inoculated on to Sabouraud Dextrose Agar with 3% glucose and 5% defibrinated sheep blood. The plates were incubated for 48 hours at 37°C. The presence of a halo around the colony indicated a positive hemolytic activity. Hemolysis was classified as type beta when

a translucent halo appeared around the inoculum, alpha type when a greenish dark halo was formed and gamma when no hemolysis was produced (20). The intensity of the production of hemolytic factor was estimated as arbitrary units, quantifying the zone diameter of the halo plus the colony in relation to colony size (Hemolytic Index = HI). The strains were classified according to the HI as negative (if HI=1.00), positive (if 1.00<HI<1.5) or strongly positive (when IH>1.5). The experiments were performed in triplicate and the results given as the mean of the values obtained.

Production of Phospholipases

Production of phospholipases was analyzed in medium that consisted of Sabouraud dextrose agar containing 1.0 M sodium chloride, 0.005M calcium chloride and 2% egg yolk (31). Plates (in triplicate) were incubated at 37ºC and the diameters of the colonies and precipitation area plus the colony were measured 7 days post inoculation for calculation of Pz (phospholipase activity zone). The Pz coefficients were grouped into 5 classes: Pz=1(negative); Pz between 0.9 and 0.99 (+), very low; Pz between 0.80 and 0.89 (+ +), low; Pz between 0.70 and 0.79 (+ + +) high; and < 0.70 (+ + + +), very high.

Protease Production

Determination of protease production was performed according to Aoki et al. (4) in medium containing bovine serum albumin (BSA; Fraction V, Sigma Chem Co., St. Louis, Mo., USA) and pH adjusted to 4.0. Plates with medium were incubated at 37ºC for 7 days. Proteinase activity was measured and calculated according to Price et al. (31). The study was repeated three times for each strain to calculate the average Pz values. The Pz coefficients were grouped into 5 classes as mentioned above.

Catalase Production

The catalase test was performed as described by Trabulsi and Altherthum (42). The *Candida* isolates were transferred to a microscope slide and a drop of 3% hydrogen peroxide was added. The immediate onset of bubbles on the surface of the suspension corresponded to a positive reaction, indicating the conversion of H2O2 into water and oxygen.

Gelatinase Production

Initially the *Candida* isolates were inoculated into BHI and after incubation at 37°C for 24 hours, the inocula were deep sown in tubes containing 5 ml of a gelatin solution at 12%, prepared in phosphate buffer at pH 7.4. After incubation at

37°C for 24 hours, the test was considered positive when gelatin liquefaction was observed (17, with adaptations).

Statistical Analysis

Data were evaluated by BioEstat 5.0. Simple frequency distribution tables were used in the univariate analysis. The association between clinical manifestations, expression of catalase, gelatinases and virulence factors in *Candida* species and between the positivity and site of origin, was determined by the chi-squared test of independence ($\chi 2$). The non parametric Kruskal-Wallis variance analysis and the Student-Newman-Keuls test were conducted for the numerical variables of phospholipases Pz, Pz of proteinases and hemolytic index. The significance level in all tests was 5%, i.e., it was considered statistically significant when $p<0.05$.

RESULTS

From a total of 144 patients, 90 patients (62.5%) had positive cultures for *Candida* spp., and the presence of yeasts was not observed in 54 patients (37.5%).

Among those 90 positive cases, clinical signs and symptoms analyzed in this research, suggestive of VVC, were clearly present in 43 cases (47.8%) and the 47 (52.2%) remaining patients were considered clinically asymptomatic. The correlation between the presence of *Candida* species and the presence/absence of symptoms was not statistically significant (x=3.22, p=0.073), as shown in Table 1.

Table 1. Association of signs and symptoms with isolates of *Candida* spp. obtained from vaginal secretions.

Species	Symptoms			χ^2	p
	Positive	Negative	Total		
C. sake	1	3	4	3.22	0.073
C. globosa	2	1	3		
C. krusei	3	1	4		
C. famata	3	2	5		
C. tropicalis	2	4	6		
C. albicans	7	7	14		
C. glabrata	7	8	15		
C. parapsilosis	18	21	39		
Total	43	47	90		

In total, eight different species belonging to the genus *Candida* (*C. parapsilosis, C. glabrata, C. albicans, C. tropicalis, C. famata, C. krusei, C. sake* and *C. globosa*) were isolated. Isolates of *C. parapsilosis* appeared more frequently totaling 43.3% (39) of the identified species, followed by *C. glabrata* with 16.7% (15), *C. albicans* with 15.6% (14) and *C. tropicalis* with 6.7% (6) (Table 2).

Table 2. Distribution of yeasts isolated from vaginal secretions of patients treated at Women's Hospital in São Luis-Ma according to species.

Candida species	N	%
C. parapsilosis	39	43.3
C. glabrata	15	16.7
C. albicans	14	15.6
C. tropicalis	6	6.7
C. famata	5	5.6
C. krusei	4	4.4
C. sake	4	4.4
C. globosa	3	3.3
Total	90	100.0

Candida samples were characterized concerning the production of hydrolytic enzymes (proteases, phospholipases, catalase, hemolysin, and gelatinase), considered virulence factors in this genus.

Among the analyzed isolates, hemolysin production was found in 72 (80%). The hemolytic activity was detected in 100% of samples of *C. albicans* and *C. tropicalis,* in 86.6% of the isolates of *C. glabrata,* followed by *C. parapsilosis* (76.9%), *C. krusei* (75%) and *C. sake* (75%). The lowest percentage of isolates per species was recorded for *C. globosa* (33.3%). The types of hemolysis observed were alpha (partial) and gamma (absence). The highest hemolytic mean index was found in *C. glabrata* isolates (1.95), followed by *C. tropicalis* (1.92) and *C. albicans* (1.79), all of them classified as strong producers of hemolysin. The difference between the means for the hemolytic indexes among species was not statistically significant (Table 3). Only two species were considered weak producers (*C. famata* and *C. globosa* isolates). The lowest hemolytic index was presented by *C. famata* with an average of 1.38.

Table 3. Hemolytic activity and Hemolytic Index of *Candida* spp isolated from vaginal secretion.

Species	N	Hemolytic Activity (%)	*Hemolytic Index
C. albicans	14	14 (100.0)	1.79
C. tropicalis	6	06 (100.0)	1.92
C. glabrata	15	13 (86.6)	1.95
C. parapsilosis	39	30 (76.9)	1.56
C. krusei	4	03 (75.0)	1.58
C.sake	4	03 (75.0)	1.55
C. famata	5	02 (40.0)	1.38
C. globosa	3	01 (33.3)	1.50
Total	90	72	

* The Hemolytic Index is a mean value of three experiments. Comparison of the mean hemolytic index between the different species of *Candida* in the vaginal area by Kruskal Wallis (H=9.7, p=0.2064) and Student-Newman-Keuh tests, showed no significant difference between them.

Phospholipase and proteinase activities were detected in 8 (8.9%) and 16 (17.8%) of the isolates, respectively (Table 4). Of the 14 tested isolates of *C. albicans*, 6 (42.8%) showed phospholipase activity. Among the tested NAC species, positive results were found in *C. glabrata* (1 positive case among 15 isolates, 6.6%) and *C. globosa* (1 case among 3, 33.3%)

The *C. famata* isolates were the largest producers of proteinase (40%), followed by *C. tropicalis* (33.3%). The *C. glabrata*, *C. parapsilosis* and *C. albicans* species showed positive activity for proteinase enzyme, but with a lower percentage (20%, 15.3% and 14.2%, respectively). Among all the analyzed isolates, the species that showed both phospholipase and proteinase activity were *C. albicans* and *C. glabrata*. Only one type of enzyme activity was recorded in the remaining species (Table 4).

Table 4. Activity of phospholipases and proteinases acid in isolates of *Candida* spp. obtained from vaginal secretions.

Species	Phospholipase			Proteinase		
	N	Positive	%	N	Positive	%
C. parapsilosis	39	0	0	39	6	15.3
C. albicans	14	6	42.8	14	2	14.2
C. tropicalis	6	0	0	06	2	33.3
C. glabrata	15	1	6.6	15	3	20.0
C. globosa	3	1	33.3	03	0	0
C. famata	5	0	0	05	2	40.0
C. krusei	4	0	0	04	0	0
C. sake	4	0	0	04	1	25.0
Total	90	8	8.9	90	16	17.8

In phospholipase activity, the minimum value of Pz analyzed was 0.90 and the highest was 0.43. In proteinase activity, the lowest value of Pz was 0.88 and the highest value was 0.47. *C. albicans* isolates exhibited an enzymatic activity considered very high (+ + + +) for phospholipase (Table 5). The activity in the *C. glabrata* isolates was also considered high (+ + +) and in *C. globosa* isolates, low (+ +). Considering the mean value of Pz for the production of the main proteinase with *Candida* isolates, we can observe that the *C. parapsilosis* species showed the largest quantity of isolates with very high enzyme activity (+ + + +). The statistical analysis performed for phospholipase showed a significant difference between the obtained mean values of Pz for *C. albicans* and *C. parapsilosis* (p=0.0005). However, the statistical analysis of proteinase activity showed no significant difference between the obtained mean values of Pz among *Candida* species (p=0.7757) (Table 5).

Most of the analyzed isolates were producers of catalase enzyme, with those of *C. glabrata* and *C. parapsilosis* being the biggest producers with 86.7% and 74.4% frequencies, respectively. The percentage of gelatinase production by *Candida* species was low. Only 4 isolates of a total of 15 *C. glabrata* and 3 of a

total of 39 *C. parapsilosis* were positive for this production. These results were statistically significant for catalase production (p=0.0045), but not for gelatinase production (p=0.4225), as shown in Table 6.

Table 5. Distribution of the value of Pz* phospholipases and proteases among isolates of different *Candida* species.

*Pz	*C. parapsilosis* n=39	*C. glabrata* n=15	*C. albicans* n=14	*C. tropicalis* n=6	*C. famata* n=5
**Phospholipase					
≤ 0.69	0	0	5	0	0
0.70-0.79	0	1	0	0	0
0.80-0.89	0	0	0	0	0
0.90-0.99	0	0	1	0	0
**Proteinase					
≤ 0.69	4	1	1	1	0
0.70-0.79	1	1	1	1	1
0.80-0.89	1	1	0	0	1
0.90-0.99	0	0	0	0	0

* Pz = phospholipase and proteinase activity area; + + + + or ≤ 0.69 = very strong, + + + or 0.70- 0.79 = strong, + + or 0.80- 0.89 = medium, + or 0.90- 0.99 = weak.
** The comparison of mean values for Pz phospholipases among different species of *Candida* from the vaginal area by Kruskal Wallis and Student-Newman-Keuh tests, showed significant difference between *C. albicans* and *C. parapsilosis* (H=26.17, p=0.0005). The comparison of mean values of Pz for acid proteinases among *Candida* spp. by Kruskal Wallis and Student-Newman-Keuh tests, showed no significant difference among them (H=4.4, p=0.7757).

Table 6. Activity of catalases and gelatinases in isolates of *Candida* spp. obtained from vaginal secretion.

Species	Catalase			Gelatinase		
	N	Positive	%	N	Positive	%
C. parapsilosis	39	29	74.4	39	3	7.7
C. albicans	14	10	71.4	14	1	7.1
C. tropicalis	06	02	33.3	06	1	16.6
C. glabrata	15	13	86.7	15	4	26.7
C. globosa	03	0	0	03	0	0
C. famata	05	01	20.0	05	0	0
C. krusei	04	01	25.0	04	0	0
C. sake	04	02	50.0	04	0	0
Total	90	58	64.4	90	9	10.0
	$\chi 2$=20.56 p=0.0045			$\chi 2$=7.06 p=0.4225		

When we considered the production of two or more virulence factors by the different species, the highest frequencies were found in *C. albicans* (n=12.85.7%), followed by *C. glabrata* (n=12.80.0%), *C. tropicalis* (n=4.66.7%) and *C. parapsilosis* (n=26.66.7%) (Figure 1). This difference was considered statistically significant (p=0.004).

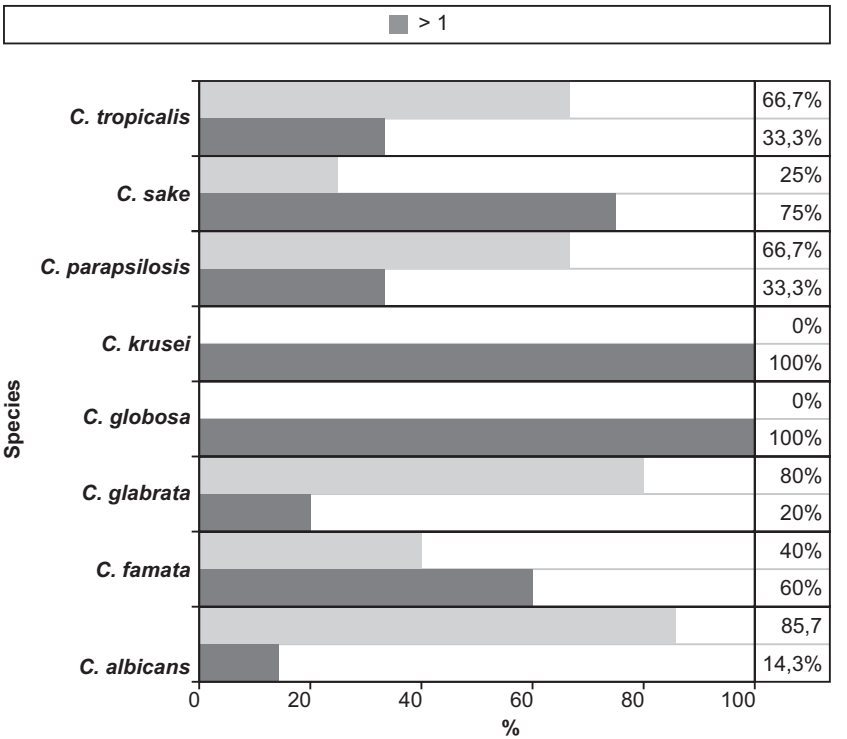

Figure 1. Percentage of isolates of each *Candida* species analyzed in relation to the amount of virulence factors produced ($\chi 2=20.8$; $p=0.004$).

DISCUSSION

Vulvovaginitis due to *Candida* yeasts (VVC) is a common condition that leads women to perform frequent medical visits in various parts of the world. It corresponds to an infectious process that ranks second among the causes of vulvovaginitis (3).

In this study, which included 144 women who voluntarily sought preventative screening for cervical cancer, the prevalence of patients with positive cultures for *Candida* yeasts was 62.5%. This result is similar to those found by Cavalcante, Miranda and Portugal (10), who obtained positive cultures for *Candida* in 61.4% of patients. This prevalence was lower than that obtained by Linhares et al. (18) with 72.7% positive cases and higher than that found by Rosa and Rumel (35) with 19.3%, and Holland et al. (16) with 46% positive cultures. These differences may have occurred because the population studied

here spontaneously sought a special service of a women's reference hospital or because of the fact that this population may have previously received an incorrect diagnosis and therefore inappropriate treatment, leading to changes in the positive culture frequencies for *Candida* (39).

Among the positive cultures, eight different species of *Candida* were identified and *C. parapsilosis* was the main species found in this study (43.3%). These results are relevant because they could indicate a changing trend in the etiology of candidiasis in women who were probably considered to have VVC, after decades of dominance of *Candida albicans* (16). These results differ from most studies reported in the literature. The literature shows much higher percentages of *C. albicans*: 96% in the Rosa and Rumel study (35) and 83.3% in the Silva et al. (38) study. *C. parapsilosis* remains an infrequent cause of fungal vulvovaginitis (41). Vaginal candidiasis is the second most common vaginal infection in the United States and Brazil, after bacterial vaginosis (2, 41) and *C. albicans* is associated with 85% to 95% of the cases, being the most prevalent species in positive cultures of secretions collected from the vaginal area (39).

In other studies, the frequency of *C. parapsilosis* also differs from the results obtained in this research: 5.3% (3) and 1.16% (45). Vaginal area yeasts may have uneven distribution. In the epidemiological study we should consider the variation according to geographical location (14), the diagnosis and the treatment used. The relative contribution of other yeast species to the increase in VVC cases is difficult to measure because vaginal yeast cultures are not routinely performed in standard VVC diagnosis (43). Due to the widespread use of oral azole and the fact that most cases of recurrent VVC are associated with non-*C. albicans* species (26) there has been an increase in non-*C. albicans* vulvovaginal cases in the United States (26, 33, 41). Thus, *Candida* isolates should be correctly identified, especially in cases of recurrent or complex vulvovaginitis so that antifungal treatment can be effective against non-*C. albicans* species (26, 33).

The percentage of patients positive for *Candida* with symptoms of VVC was not higher than the asymptomatic ones and a relationship between symptoms and isolated species was not established. In agreement with our findings are the Rose and Rumel (35) and the Boatto et al. (7) findings, where no correlation between signs/symptoms and positive culture for *Candida* was found.

Regarding the assessment of hemolytic factor production, all eight *Candida* species showed some type of hemolytic activity (80% of isolates), indicating the ability of these isolates to exploit potential iron sources. This percentage is close to that obtained in the Luo, Samaranayake and Yau (19) study, where the overall prevalence of evidence of hemolytic activity was 81.3% among species from various sources. The authors recorded the highest percentages of hemolysis in the species *C. albicans* and *C. tropicalis* (100%). The Negri et al. (25) trial involving 27 *C. albicans* nosocomial strains, observed total hemolysis in all strains and França et al. (15) observed 100% hemolysis in isolates of *C. tropicalis*. In the study of Rörig et al. (34), only *C. albicans* and *C. parapsilosis* had hemolytic activity. In our study,

no statistically significant difference was found (p=0.2064) between the means of the hemolytic indices and analyzed *Candida* species. The highest hemolytic average index in *Candida* isolates evaluated in this study (1.95) was lower than that obtained in the study of Luo et al. (19), which was 2.22. The hemolytic activity of medically important yeasts like *Candida* has rarely been explored. Data from this study are important because they show that the majority of vaginal secretion isolates have the ability to use iron derived from hemoglobin through the production of a factor responsible for the lysis of erythrocytes, and this ability may represent an adaptive advantage for the establishment of infections in their hosts (21).

The percentages of phospholipase and proteinase enzyme activity among isolates of non-*albicans* species were low, equivalent to 8.9% and 17.8%, respectively. Among the *C. albicans* isolates only 6 of 14 (42.8%) were positive for phospholipase and 2 of 14 (14.2%) for proteinase. Regarding the tested isolates of *C. parapsilosis*, the most prevalent species, only 6 of the 39 were positive for proteinase and none were positive for phospholipase. However, four of those showed a positive proteinase activity considered to be high (Pz<0.69). Furthermore, when considering all species together, the isolates were more regular producers of proteinases (16 of 90) than phospholipases (8 of 90).

These results differ from some of the literature such as Camargo et al. (8) that found 58.3% positive samples for proteinase activity and 68.8% for phospholipase among *Candida* isolates obtained from vaginal discharge, and those of Candido et al. (9) that show a positive frequency of 71.9% for phospholipase and 68.7% for proteinase in *Candida albicans* strains isolated from the vaginal mucosa. In the test performed by Shinobu et al. (37), proteins were also significantly expressed by *C. albicans* isolated from vaginal secretions.

On the NAC species our results are partly compatible with those of Camargo et al. (8) where they found that these species showed no proteinase activity and only one isolate of *C. tropicalis* demonstrated the production of phospholipase, with Pz of 0.73. According to these authors, these results would be expected, since the non-*albicans* species such as *C. krusei* and *C. glabrata* are not producers of these enzymes. However, other authors such as Panizo et al. (28), Mohan and Ballal (24), D'Eça Jr et al. (13) have shown that some species of NAC such as *C. glabrata*, *C. tropicalis, C. sake* and *C. parapsilosis* can secrete phospholipases and proteinases.

A small percentage of isolates produced gelatinase (10%), meanwhile, the production of the catalase enzyme was significant for the most prevalent species. Twenty nine *C. parapsilosis* isolates were catalase producers (74.4%), which is interesting since catalase degrades hydrogen peroxide, a byproduct of metabolism of *Lactobacillus* genus bacteria, the main microorganism that controls the proliferation of *Candida* in the vagina (30).

When we considered the production of two or more virulence factors among species, *C. albicans* stood out by presenting an 85.7% frequency of producer isolates and this difference was statistically significant compared to the other species (p=0.004) thus proving the higher pathogenic power of this species.

Although *C. parapsilosis*' links to vulvovaginitis remain debated (26, 33), Agatensi et al. (1) have already verified that *C. parapsilosis* isolates from women with vulvovaginitis secrete more aspartyl proteinases *in vitro* than those isolated from asymptomatic patients. This was not observed in our study. However, these same strains were good producers of catalase and hemolytic factor, the last being an important virulence factor because it could compromise the normal integrity of the vaginal cells by hydrolyzing some membrane proteins, besides affecting the host's defense mechanisms, contributing to the pathogenic capacity of the analyzed isolates (19). Pendrak et al. (29) suggest that hemolytic activity may also be involved in the mechanism of phenotypic changes, related to induction of a more virulent phenotype or resistance to the body's defenses. The production of catalase could favor the survival and proliferation of yeast in a predominantly bacterial vaginal environment, or these strains may be producing other factors or virulence properties that may contribute to the establishment of the VVC but were not evaluated in this study. In fact, *Candida* strains have different virulence factors to colonize the vagina and stay at this site. The presence of these factors may be related to the onset of the disease and the development of the infection or strongly contribute to the infection itself (27). Furthermore, in our results *C. parapsilosis* proteinase producers were detected in both symptomatic and asymptomatic patients. The same results were verified in relation to the other enzymes analyzed. Statistical analysis of the production of each virulence factor for the presence or absence of symptoms was not significant. Taken together these data show the relevance of other factors, such as those of hosts and environmental factors on the emergence of *Candida* vulvovaginitis. Moreover, it is concluded that signs and symptoms are presumptive because they did not correlate with the isolated species.

CONCLUSIONS

From a total of 144 patients, 90 patients (62.5%) had positive cultures for *Candida* spp. with *C. parapsilosis* the most frequent species. Clinical signs and symptoms suggestive of VVC, were clearly present in 43 cases (47.8%), although there was no correlation between the presence of *Candida* species and the presence/absence of symptoms. Most of the analyzed isolates were good producers of hemolysin and catalase enzymes. The percentage of phospholipase, proteinase and gelatinase production by *Candida* species was low, in relation to the production of two or more virulence factors by the different species, the highest frequencies were found in *C. albicans* isolates.

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